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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	6
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	10
References.....	11
Appendices.....	12

INTRODUCTION:

Prostate Cancer Metastasis.

Our ability to detect and successfully treat localized prostate cancer (PC) has improved appreciably in recent years. However, metastatic disease presents a continuing therapeutic challenge and still represents the most common cause of PC-related death. The symptomatic phase in PC is largely due to the presence of metastasis, which leads to painful bone disease and often numerous clinical complications. Once metastasis has occurred, 70% of these patients will die of PC rather than an unrelated cause (1). Thus, an urgent need for novel diagnostic and/or predictive measures is needed to curtail disease progression. The current screening methods for early-stage disease, including conventional and histological techniques, are limited by their inability to predict accurately the true extent and prognosis of a substantial proportion of clinically localized cancers (2-4). This limitation is due, in part, to inherent limitations and subjectivity of current grading and staging systems (5, 6). The relapse rate after radical retroperitoneal prostatectomy in PC patients with favorable prognosis (i.e. well-differentiated histology, low PSA) has approached 20-30% (7-9). Another study demonstrated that 20 to 57% of men with histologically confined disease who underwent radical prostatectomy developed an elevation of PSA, suggesting presence of micrometastasis (10, 11). Even in patients with small tumors and tumor-negative lymph nodes (T1N0), there is a 15-20% likelihood of distant metastasis (12). Overall, it is clear there is a critical need for markers that will distinguish with accuracy those histological lesions and disseminated cells associated with clinical metastatic disease from those that remain indolent (6, 13).

Molecular Basis of PC Metastasis.

Although metastasis is the most lethal attribute of PC, the underlying molecular mechanisms have not been delineated. PC tumor growth at the site of metastasis (e.g. bone) is an important clinical target, since cells must survive and proliferate to form overt clonal expansion and macroscopic lesions. The molecular events that underlie the stepwise development from normal cells, via metaplasia, dysplasia, and carcinoma *in situ*, to a localized tumor and ultimately metastasis are still fragmentary (14-16). Thus, the first step toward developing therapies to inhibit such growth is to identify the *genes* that regulate metastatic colonization. Prostate tumors are frequently found to be multifocal and their cellular composition is heterogeneous (14-16). The molecular changes leading to metastasis in one clone of cells may be obscured by various molecular events occurring simultaneously in other cells of same tumor but not in the metastatic pathway (14). Detection of such alterations in gene activities related to metastasis when analyzed by subtractive hybridization and differential display will yield information with high precision. This is contingent, however, on the nature of the RNA pools used for comparison. Because most of previous studies have compared pools of RNAs of normal to metastatic cells, high misleading results were obtained. Another problem that can be misleading stems from the fact that whole specimens, rather than pure microdissected tumor cells, are used for comparison. To get a step closer to identifying human PC metastasis-related genes, we have resolved the first problem by comparing primary and metastatic RNA pools, and the second problem by procuring pure cell populations using laser capture microdissection (LCM). Using this approach, we were able to identify at least twenty metastasis-related genes, two of which were found to be novel.

Prostate Cancer Metastasis-Related Genes.

It is conceivable that genetic alterations in specific genes that control or affect multiple biological activities and molecular pathways generate cells predisposed to metastasis. Mutations in these “control genes” could accelerate progression to full metastatic phenotype (17). The loss or aberrant activities of specific genes that regulate transcription may account in part for hundreds of alterations in gene expression observed in malignancies (18-21), and could underlie the abrupt transition to metastatic phenotype that are observed clinically in PC (22-23). *In PC, only a few genes have been found to be related to metastasis.* This may be attributed to difficulty of obtaining biopsy specimens from advance disease patients and the lack of appropriate animal models. In broad context, metastasis control genes, such as p53 and DNA mismatch repair genes, may also regulate cell proliferation and tumorigenesis. In PC, mutations of p53 gene have been shown to be more frequent in metastases compared with primary tumors (24-26). In addition, caveolin-1, has been found to be associated with PC metastasis (14). However, it should be emphasized that most of these studies have generated

data based on non-microdissected specimens– indicating that such comparative analyses based on inappropriate pools of mRNA may yield highly misleading results. This is complicated by tumor cell heterogeneity and the presence of expressed sequences that are not related to metastasis. Thus, further studies are needed to identify and characterize gene(s) involved in development of PC metastasis.

Limitations of Current Techniques.

The elucidation of the genetic events underlying the initiation and progression of human prostate cancer has been hampered by the limitations inherent to both *in vitro* and *in vivo* methods of study. The most significant limitation of the *in vitro*-based systems is that the genetic information derived from cell lines may not be representative of the molecular episodes occurring in the tissue microenvironment from which they were derived. In addition, the *in vivo* genetic analysis of PC has been restricted by our inability to secure an unadulterated cell population from the complex heterogeneous tissue. Although a number of studies have been conducted with *in vitro*-derived genetic materials from both mammalian and nonmammalian systems (27-29), a major leap in functional genomic investigation would be the ability to perform genetic subtractive analysis with *in vivo*-derived genetic material originating from a morphologically distinct cellular subpopulation within neoplastic tissue. These limitations have been overcome by the recent advent of LCM, a new technology for procuring pure cells from specific microscopic regions of tissue sections (30). Cell types undergoing similar molecular changes, such as those thought to be most definitive of the disease progression, may constitute less than 5% of the volume of the tissue biopsy sample. Therefore, LCM is critical to the application of molecular analysis of genes in actual tissues.

Recently, several methods have been designed to detect and isolate different DNA sequences present in one complimentary (31) or genomic (32) DNA library but absent in another. The advent of suppressive hybridization technique (SSH) technique, with its capacity to simultaneously analyze several genes, provides a unique tool for high-throughput genetic analysis of cancer (33-35). Initially, subtractive hybridization using conventional methods has met with some success, which has led to a series of developments in which PCR has been applied (36-38). SSH enhances the probability of identifying increased expression of low-abundance transcripts and represents an advantage over other methods of identifying differentially expressed genes, such as differential display-PCR (39) and cDNA representation difference analysis (32). While this method could have been powerful in elucidating differentially expressed genes in many disorders if applied correctly, the use of control specimens different from the native tissue for subtractive genomic analysis in some studies has created many inconclusive results. Cell to cell or procuring tester and driver cells from the same patient or animal using the LCM technique can minimize homologous sequence variation *in vivo*.

BODY:

Task 1: To characterize Seq1 and Seq2 genes (months 1-12):

a. Determination of full-length cDNAs of Seq 1 and Seq 2 genes.

We have characterized the full-length cDNAs of the Seq1 and Seq2 genes using at least two 5' and 3' rapid amplification of cDNA ends (RACE) commercial kits (Invitrogen Carlsbad, CA, and Seegene, Rockville, MD). Because of unique secondary structures and low transcript levels of these genes in prostate cancer cell lines, we had great deal of difficulty in accomplishing this task in a timely fashion. We have spent considerable amount of time using one commercial kit at a time trying to unveil the full-length cDNA sequences of the two genes. We have also designed several sets of nested GSPs to verify our cloned genes. To enrich the cDNAs, we have modified the cDNA synthesis step using different approaches. We obtained measurable cDNA levels for both 5' and 3' RACE using a modified SMART cDNA synthesis technique (Clontech) and Superscript reverse transcriptase kit (Invitrogen) under stringent conditions as described in table 1.

Table 1: Modified SMART cDNA synthesis (RT) Protocol for RACE

	<u>5'-RACE</u>	<u>3'-RACE</u>
Total RNA (~ 4 µg /µl)	1 µl	1 µl
5'-SMART II CDS primer II A (12 µM)	1 µl	—
SMART IIA oligonucleotide (12 µM)	1 µl	—
3'-Smart CDS primer (12 µM)	—	1 µl
DEPC deionized water	<u>9 µl</u>	<u>10 µl</u>
Total volume	12 µl	12 µl
Mix contents, centrifuge briefly, heat mixture @ 70°C for 2 min in thermal cyclor. Incubate on ice water for 2 min. Add the following to each tube:		
5X First-strand buffer	4 µl	4 µl
0.1 M DDT	1 µl	1 µl
RNAzin (40 U/µl)	1 µl	1 µl
dNTP mix (10 mM each)	1 µl	1 µl
SuperScript™ III RT (200 U/µl)	<u>1 µl</u>	<u>1 µl</u>
Total volume	20 µl	20 µl

Incubate at 55°C for 3.0 hr in a thermal cyclor.

For 3' and 5' RACE PCR amplifications of each commercial kit, we optimized the PCR conditions for each kit by designing several sets of gene-specific primers (GSP; 23-28 nt long) with 50-70% GC and T_m of 55 to 75°C for each gene. The unique secondary structures, high GC content, short SSH sequences, and low levels of expression of these genes in prostate cancer cell lines were problematic. Because we know only fragment sequences of these genes (~ 200), we spent considerable amount of time designing primers that would amplify the 5' and 3' ends of the genes using various commercial kits. The primers were tested for amplification of our target genes in transcripts isolated from prostate cancer cell lines, PC-3 and DU-45 cells (Table 2). The major limitation of our approach was the short known sequences of the target genes (~ 200 bp) and hence our limitation in finding appropriate set of PCR primers that match suggested length, GC content and T_m of the anchor primers (3' and 5') of each RACE kit. Despite our initial successful cDNA synthesis attempts of both genes using SMART II CDS primer, SMART IIA oligonucleotide and SuperScript™ III RT (Table 1), we were unable to amplify target genes using multiple sets (ie lengths, T_m, GC contents) of gene specific primers under various conditions by a PCR standard technique (Table 2).

Table 2: 5' & 3' RACE Initial PCR Reaction

	1	2	3	4	5	6	7	8
5' cDNA synthesis rxn	1µl		1µl	1µl				
3' cDNA synthesis rxn		1µl			1µl	1µl		
Seq1-2 plasmid (100 ng/µl)							1µl	
Seq1-4 plasmid (100 ng/µl)								1µl
GAPDH primer (U) (10 µM)	1µl	1µl						
GAPDH primer (L) (10 µM)	1µl	1µl						
GSP1 (Seq1-2)			1µl		1µl		1µl	
GSP2 (Seq1-2)			1µl		1µl		1µl	
GSP1 (Seq1-4)				1µl		1µl		1µl
GSP2 (Seq1-4)				1µl		1µl		1µl
PCR SuperMix (Invitrogen)	5µl	5µl	5µl	5µl	5µl	5µl	5µl	5µl
H2O2	42µl	42µl	42µl	42µl	42µl	42µl	42µl	42µl
Total	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl

Because of the limitations of the gene specific primer sequences we can design and that failure of PCR amplification may be attributed to high GC content of the target genes, we used a PCR amplification protocol based on Advantage GC-2 polymerase mix as described in Table 3. The results demonstrate successful amplification of both genes (Fig 1) and further confirmed our assumption of the high GC content of both genes.

Table 3: Rapid Amplification of cDNA Ends (RACE)

Component	Seq1-2 5'-RACE Sample	Seq1-2 3'-RACE Sample	Seq1-4 5'-RACE Sample	Seq1-4 3'-RACE Sample
5'-RACE ready cDNA	2 µl		2 µl	
3'-RACE ready cDNA		2 µl		2 µl
UPM (10 x)	1 µl	1 µl	1 µl	1 µl
Seq1-2 GSP1 (10 µM)	1 µl			
Seq1-2 GSP2 (10 µM)		1 µl		
Seq1-4 GSP1 (10 µM)			1 µl	
Seq1-4 GSP2 (10 µM)				1 µl
5 X GC2 PCR Buffer	10 µl	10 µl	10 µl	10 µl
GC melt (5 M)	5 µl	5 µl	5 µl	5 µl
dNTP mix (10 mM)	1 µl	1 µl	1 µl	1 µl
AdvantageGC-2Polymerase mix	1 µl	1 µl	1 µl	1 µl
Nuclease-free water	29 µl	29 µl	29 µl	29 µl
Total	50 µl	50 µl	50 µl	50 µl

We had difficulty amplifying the '3 and 5' RACE products of our target genes using either Invitrogen or Clontech kits with or without an Advantage GC-2 polymerase. We finally used a modified SeeGene DNA Walking SpeedUP approach with four DW-ACP primers in conjunction with the Advantage GC-2 polymerase kit in three PCR steps as described below.

Modified SeeGene PCR-1		
PCR Mix		
DU-145 cDNA		1 µl
5 X GC-2 PCR Buffer		10 µl
GC Melt (5 M)		5 µl
DW-ACP 1, 2, 3 or 4 (2.5 µM)		4 µl
Target Specific Primer-1 (TSP-1) (10 µM)		1 µl
dNTP mix (10 mM)		1 µl
Advantage GC-2 Polymerase mix		1 µl
Nuclease-free water		<u>27 µl</u>
Total volume		50 µl
Preheat thermal cycler to 94°C		
PCR Cycles		
<u>1 Cycle:</u>	94°C	5 min
	42°C	1 min
	72°C	2 min
<u>35 Cycles:</u>		
	94°C	40 sec
	55°C	40 sec
	68°C	2.5 min
<u>1 Cycle:</u>		
	68°C	3 min
Purify PCR products using Quiagen column kit (use 30 µl elution volume)		

Modified SeeGene PCR-2		
PCR Mix		
First PCR products		2 µl
5 X GC-2 PCR Buffer		10 µl
GC Melt (5 M)	5 µl	
DW-ACP-N (10 µM)		1 µl
Target Specific Primer-2 (TSP-2) (10 µM)	1 µl	
dNTP mix (10 mM)		1 µl
Advantage GC-2 Polymerase mix	1 µl	
Nuclease-free water		<u>29 µl</u>
Total volume		50 µl
PCR Cycles: Important preheat thermal cycler to 94°C		
1 Cycle:	94°C	3 min
35 Cycles:	94°C	40 sec
	60°C	40 sec
	72°C	90 sec
1 Cycle: 72°C		7 min

Modified SeeGene PCR-3		
PCR Mix		
First PCR products		2 μ l
5 X GC-2 PCR Buffer		10 μ l
GC Melt (5 M)		5 μ l
Universal Primer 10 μ M)		1 μ l
Target Specific Primer-3 (TSP-3) (10 μ M)		1 μ l
dNTP mix (10 mM)		1 μ l
Advantage GC-2 Polymerase mix		1 μ l
Nuclease-free water		<u>29 μl</u>
Total volume		50 μ l
PCR Cycles: Important preheat thermal cyclers to 94°C		
<u>1 Cycle:</u>		
94°C		3 min
<u>40 Cycles:</u>		
94 °C		40 sec
63 °C		40 sec
72 °C		90 sec
<u>1 Cycle:</u>		
72 °C		7 min

With this approach, we were able to amplify the 5' and 3' PCAE products of our target genes as shown in Fig1.

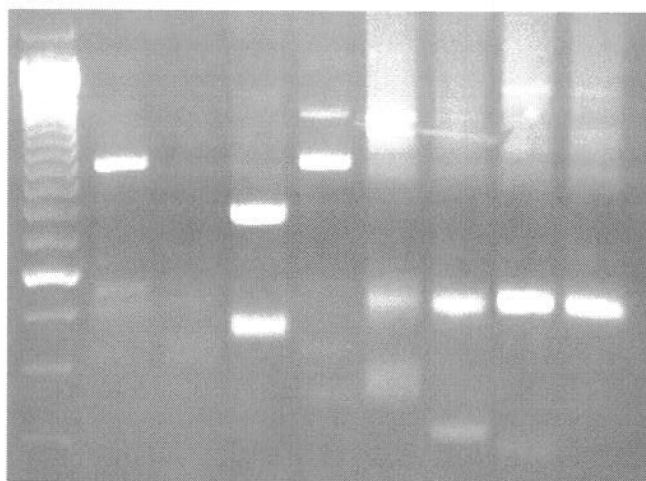


Fig 1: Example of 5' and 3' RACE amplification of target genes. Lane 1, Marker, lanes 2-4, Seq1 gene 5' RACE using DW-ACP primer 1, 2, 3 and 4, lanes 5-8, Seq1 gene 3' RACE using DW-ACP primer 1, 2, 3 and 4.

The PCR products were sequenced matched with Seq1 and Seq2 genes and cDNAs were cloned in expression plasmids using standard protocols. The expression plasmids will be used for production of the recombinant proteins and antibodies.

KEY RESEARCH ACCOMPLISHMENTS:

- Optimized conditions for amplification of the target genes.
- 3' and 5' RACE amplification of target genes.

REPORTABLE OUTCOMES:

No reportable outcome at this stage of the proposed research protocol.

CONCLUSIONS:

We propose to identify the functional characterization of two novel cancer-specific, metastasis-related genes whose constitutive expression may be pivotal for prostate cancer progression. Work accomplished was performed based on the proposed statement of work. We have characterized the full-length cDNAs of the Seq1 and Seq2 genes using at least three 5' and 3' rapid amplification of cDNA ends (RACE) commercial kits (Invitrogen Carlsbad, CA, BD Bioscience (Clontech Inc), and Seegene, Rockville, MD). To optimize the PCR conditions for each kit, we had designed several sets of gene-specific primers (GSP; 23-28 nt long) with 50-70% GC and T_m of 55 to 75°C for each gene. We have also designed several sets of nested GSPs to verify our cloned genes. Because of unique secondary structures, high GC content, short SSH sequences, and low levels of expression of these genes in prostate cancer cell lines, we had great deal of difficulty in accomplishing this task in a timely fashion. As such, we devised different strategies for the first-strand synthesis using a modified oligo(dT) primers (5'-CDS primer or 3'-CDS primer), and Smart oligo II primer under various conditions. The full-length cDNA sequences were subcloned into mammalian expression vectors (Invitrogen) and ready to be used for generation of recombinant proteins and antibody production.

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APPENDICES: No materials attached to this report. Refer to data in Body Section.